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Identification of an ovine atadenovirus gene whose product activates the viral E2 promoter: possible involvement of E2F-1

Daniel Kümin,^{a,1} Christian Hofmann,^{a,2} Wolfgang Uckert,^b
Gerald W. Both,^{c,*} and Peter Löser^{a,b,3}

^a *Develogen AG, 37079 Göttingen, Germany*^b *Humboldt University Berlin, Institute of Biology, Molecular Cell Biology and Gene Therapy, MDC Berlin, 13092 Berlin, Germany*^c *Molecular Science, CSIRO, North Ryde, New South Wales 2113, Australia*

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Abstract

Activation of the adenoviral E2 promoter is an early step in adenovirus gene expression. For members of the mast- and aviadenoviruses, this requires induction of the cellular transcription factor E2F by virally encoded gene products such as E1A, E4orf6/7 and orf22/GAM-1. The newly recognized genus atadenovirus, of which the ovine isolate OAdV is the prototype, lacks any sequence homology to those genes. To find a possible link between E2 promoter activation and OAdV gene expression, we utilized a screening method to search for genes within the OAdV genome that were capable of stimulating the viral E2 promoter. One such gene, E43, was identified within the proposed E4 region toward the right-hand end of the OAdV genome. The E43 gene product was also found to be capable of stimulating E2F-1-dependent gene expression. A closer inspection of the E2 promoter revealed the presence of a non-palindromic E2F binding site within the OAdV E2 promoter. Mutation of this site markedly reduced both E2F-1- and E43-dependent promoter activation. Moreover, a direct protein–protein interaction of the E43 gene product with E2F, but not with the retinoblastoma protein pRb, suggested a possible cooperation between these two proteins in activating the E2 promoter. The importance of the E43 gene product for virus replication is also underlined by the finding that an OAdV recombinant with a functionally inactivated E43 gene showed severely inhibited virus growth.

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Introduction

Ovine adenovirus type 7 (isolate 287, OAdV) (Boyle et al., 1994) is the prototype of the atadenoviruses, a genus that was formally recognized by the ICTV in 2002. The genus also includes egg drop syndrome virus, several subtypes of bovine adenovirus (e.g. BAdV-4) and other animal isolates including those from reptiles

(Benkö and Harrach, 1998; Dan et al., 1998; Farkas et al., 2002; Harrach and Benkö, 1999; Harrach et al., 1997; Thomson et al., 2002; reviewed in Both, 2001). These viruses are distinguished from other adenovirus genera by a distinct genome organization and by the fact that many open reading frames show no detectable homology to those of any other adenoviruses (Venkatesh et al., 1998; Vrati et al., 1995, 1996a,b). Because OAdV has been successfully developed as a gene delivery vector (Hofmann et al., 1999; Khatri et al., 1997; Löser et al., 1999, 2000; Voeks et al., 2002; Xu et al., 1997; reviewed in Both, 2002a; Löser et al., 2002) and all viral genes are retained in many of these vectors, it is important to know more about their function.

The transcription of adenoviral E2 genes, which code for proteins required for viral DNA replication, is an early step in the adenovirus life cycle. In mast- and aviadenoviruses, activation of these genes involves the

* Corresponding author. Molecular Science, CSIRO, PO Box 184, North Ryde, New South Wales 1670, Australia. Fax: +61-2-9490-5005.

E-mail address: gerry.both@csiro.au (G.W. Both).

¹ Present address: Institute of Veterinary Virology, University of Bern, Länggass-Str. 122, Bern, Switzerland.

² Present address: Berolina Drug Development GmbH, Bitterfelder Str. 19, 12681 Berlin, Germany.

³ Present address: Robert Koch Institute, Seestr. 10, 13353 Berlin, Germany.

induction of transcription factors of the E2F family. In particular, in certain mastadenoviruses (such as human adenovirus type 5), products of the E1A and E4 region are involved in activation of the E2F factors. The E1A gene is the first gene to be expressed during an adenovirus infection (reviewed in Shenk, 1996) and its gene products override normal regulatory constraints at the G1/S boundary and induce expression from several viral promoters. One cellular target of E1A that is involved in cell cycle control is the retinoblastoma protein (pRb). E1A proteins bind to the hypophosphorylated form of pRb, resulting in the formation of a stable complex between E1A and pRb and the release of free, presumably transcriptionally active E2F (reviewed in Nevins, 1995). On the other hand, the adenovirus E4orf6/7 product binds to E2F, promotes E2F dimer formation and directs such complexes to binding sites within the HAdV5 E2 and cellular E2F-dependent promoters where they bind with increased stability. The induction of stabilized E2F binding correlates with the transcriptional activation of these promoters (Hardy and Shenk, 1989; Hardy et al., 1989; Huang and Hearing, 1989; Marton et al., 1990; Neill and Nevins, 1991; Neill et al., 1990; Obert et al., 1994; O'Connor and Hearing, 1991; Reichel et al., 1989; Schaley et al., 2000). Thus, E1A and E4orf6/7 utilize complementary mechanisms for enhancing the expression of E2F-dependent genes, including those involved in cell cycle progression. E2F modulating activity was also described for the GAM-1 and orf-22 gene products of CELO, the prototype of the aviadenovirus genus (Lehrmann and Cotten, 1999).

Members of the atadenovirus genus (typified by OAdV) do not show any sequence homology to the mast- and aviadenovirus genes whose products interfere with the E2F pathway. Specifically, OAdV lacks a typical E1 region. Instead, the left-hand region of the OAdV genome codes for a structural protein (p32K) and three open reading frames whose protein products have not been formally identified. At the opposite end, the proposed E4 region is located at the penultimate transcription unit and appears to code for three putative proteins whose functions are still unknown (reviewed in Both, 2001). At the extreme right end is the RH transcription unit that codes for a family of related F-box proteins. These have the potential to act as modulators of a cellular pathway(s) which is as yet unidentified (Both, 2002b). We were therefore interested to determine whether OAdV protein(s) coded for by the LH, RH or E4 regions could participate in the activation of the viral E2 promoter and whether this activation might involve E2F transcription factors of the ovine host cell. In the study presented, we have identified one such gene, E43, the product of which activated transcription from the E2 promoter. The E43 gene product also stimulated transcription from a solely E2F-dependent promoter and

directly interacted with E2F-1. In addition, functional inactivation of the E43 gene in the OAdV genome severely inhibited virus growth, underlining the importance of this gene product for virus replication.

Results

Identification of an OAdV gene product that stimulates the E2 promoter

Initially, we wished to determine whether infection with OAdV resulted in an activation of the autologous OAdV E2 promoter. For this purpose, the OAdV E2 promoter (nt 21,639–21,335 of the OAdV genome) was cloned in front of the firefly luciferase reporter gene to produce pOAdVE2-luc (Fig. 1A). This plasmid was used to transfect CSL503 cells that are permissive for OAdV. After 4 h, cells were infected with increasing amounts of OAdV. At 30 h post-infection, cells were harvested and reporter gene expression was assayed (Fig. 1B). Infection with OAdV resulted in a significant increase (up to 4.8-fold over non-transfected controls) in reporter gene activity. This indicated that OAdV might contain genes whose products can activate the E2 promoter.

To test this hypothesis and to exclude that activation of the OAdV E2 promoter was due to processes independent of viral gene expression, we cloned several, partially overlapping sub-regions of the OAdV genome (Fig. 2A). These regions spanned the left- and right-hand ends of the OAdV genome that contain genes of unknown functions. The resulting plasmids were co-transfected together with pOAdVE2-luc into CSL503 cells (Fig. 2B). Whereas the plasmid containing the left-hand end *Bam*HI fragment of the OAdV genome (pOAdV-B1, carrying p32 and the open reading frames LH1, 2 and 3) did not activate luciferase gene expression from the E2 promoter, the right-hand end *Bam*HI fragment (pOAdV-B6) carrying the genes of the putative E4 and RH region clearly activated luciferase gene expression. A similar activation was observed with the right terminal *Hpa*I fragment of the viral genome (pOAdV-H8), which also contained these regions. A refinement of this approach showed that a plasmid containing the putative E4 region of OAdV (pOAdV-E4), but not a plasmid carrying the RH region (pOAdV-RH), was able to activate the promoter. Thus, the gene function capable of stimulating the OAdV E2 promoter appeared to reside within the proposed E4 region of the virus.

The OAdV E4 region consists of three open reading frames (E41, E42 and E43) all of which are expressed in OAdV-infected permissive cells as shown by immunofluorescence and Western blotting experiments using polyclonal rabbit sera raised against the individual E4 products (data not shown). To determine which of the E4 proteins was responsible for activation of the E2

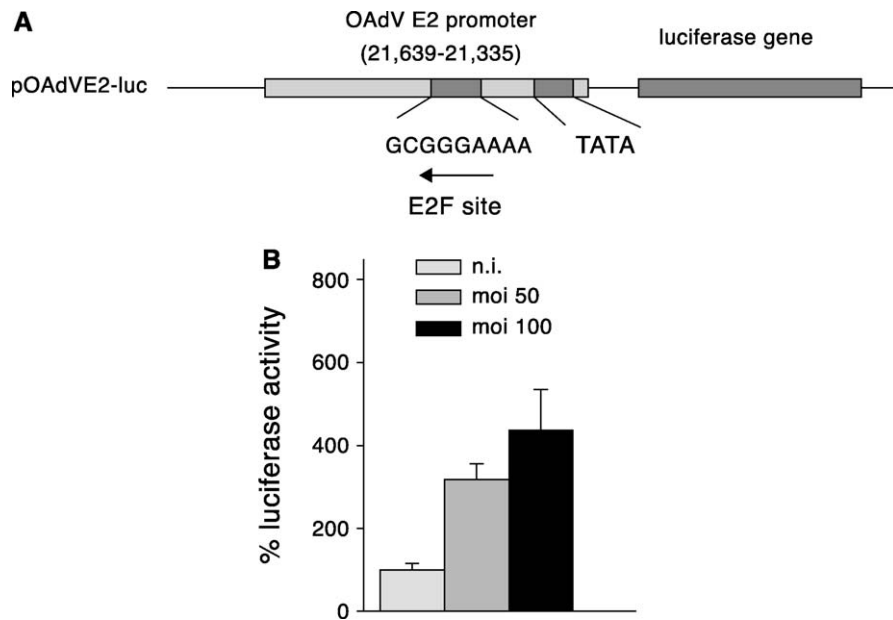


Fig. 1. Activation of the OAdV E2 promoter upon OAdV infection. (A) Scheme of plasmid pOAdVE2-luc containing the E2 promoter with the non-palindromic E2F binding site. (B) Reporter plasmid pOAdVE2-luc (0.8 μ g) and transfection control plasmid pRSV-lacZ (0.2 μ g) were co-transfected into CSL503 cells. At 4 h post-transfection, cells were infected with OAdV at the multiplicity of infection (moi) indicated. At 30 h post-infection, cells were harvested and reporter gene activities were determined. The ratio of luciferase to lacZ gene activity is shown with reporter gene activity ratio in uninfected cells set at 100%. Error bars indicate standard deviations from four independent experiments. n.i., non-infected cells.

promoter, we PCR-cloned the individual ORFs into an expression vector (see Materials and methods). The resulting plasmids (pCI-E41, pCI-E42 or pCI-E43) were co-transfected together with pOAdVE2-luc into CSL503 cells (Fig. 2C). Whereas co-expression of E41 and E42 did not increase reporter gene activity, a significant activation of E2 promoter-dependent expression was observed with the E43 plasmid (3.2-fold over control). This clearly indicated that the OAdV E43 gene product could activate the viral E2 promoter.

The E43 gene product activates E2F-dependent gene expression

Activation of mastadenovirus E2 promoters (e.g. those of HAdV2 and 5) involves induction of E2F transcription factors following viral infection. To test whether E2F might be involved in activating the E2 promoter of OAdV (and probably other members of the atadenovirus genus that have sequence homology in this region), we performed transfection experiments with plasmid pE2F-luc that contained three palindromic E2F binding sites linked to a TATA box in front of the firefly luciferase reporter gene (Fig. 3). In this plasmid, luciferase gene expression was solely dependent on activated cellular E2F (see below). Transfection of this plasmid into CSL503 cells followed by infection with OAdV as described for Fig. 1 resulted in a 6.8-fold stimulation of luciferase activity, indicating that OAdV infection caused an increase in transcriptionally active E2F (Fig. 3A).

To determine which products might be involved in the observed E2F activation, we co-transfected plasmids containing overlapping OAdV fragments of the OAdV genome together with pE2F-luc into CSL503 cells. Co-transfection resulted in a modest increase (1.8- to 2.4-fold over control) in luciferase activity when plasmids were used that contained the OAdV E4 region (pOAdV-B6, pOAdV-H8 and pOAdV-E4), but not with the other plasmids (pOAdV-B1, pOAdV-RH, data not shown). Co-transfection of expression plasmids coding for an individual E4 gene together with pE2F-luc caused a strong increase in luciferase gene expression when the E43 gene was used (5.9-fold over control), whereas expression of E41 or E42 had no effect on luciferase gene expression (Fig. 3B). Since the stimulation of luciferase expression from pE2F-luc was absolutely dependent on E2F (see below), the E43 gene product clearly induced cellular E2F activity.

E2F activates expression from the OAdV E2 promoter

The E2 promoter of OAdV contains a single, non-palindromic E2F site (Fig. 1A). To test whether E2F was involved in activation of the E2 promoter, we co-transfected pOAdVE2-luc together with an expression plasmid for human E2F-1. E2F-1 co-expression strongly stimulated the OAdV E2 promoter in a dose-dependent manner up to 6.8-fold over control. In a positive control experiment, luciferase expression from the artificial E2F-dependent promoter (pE2F-luc) was 19.7-fold elevated with

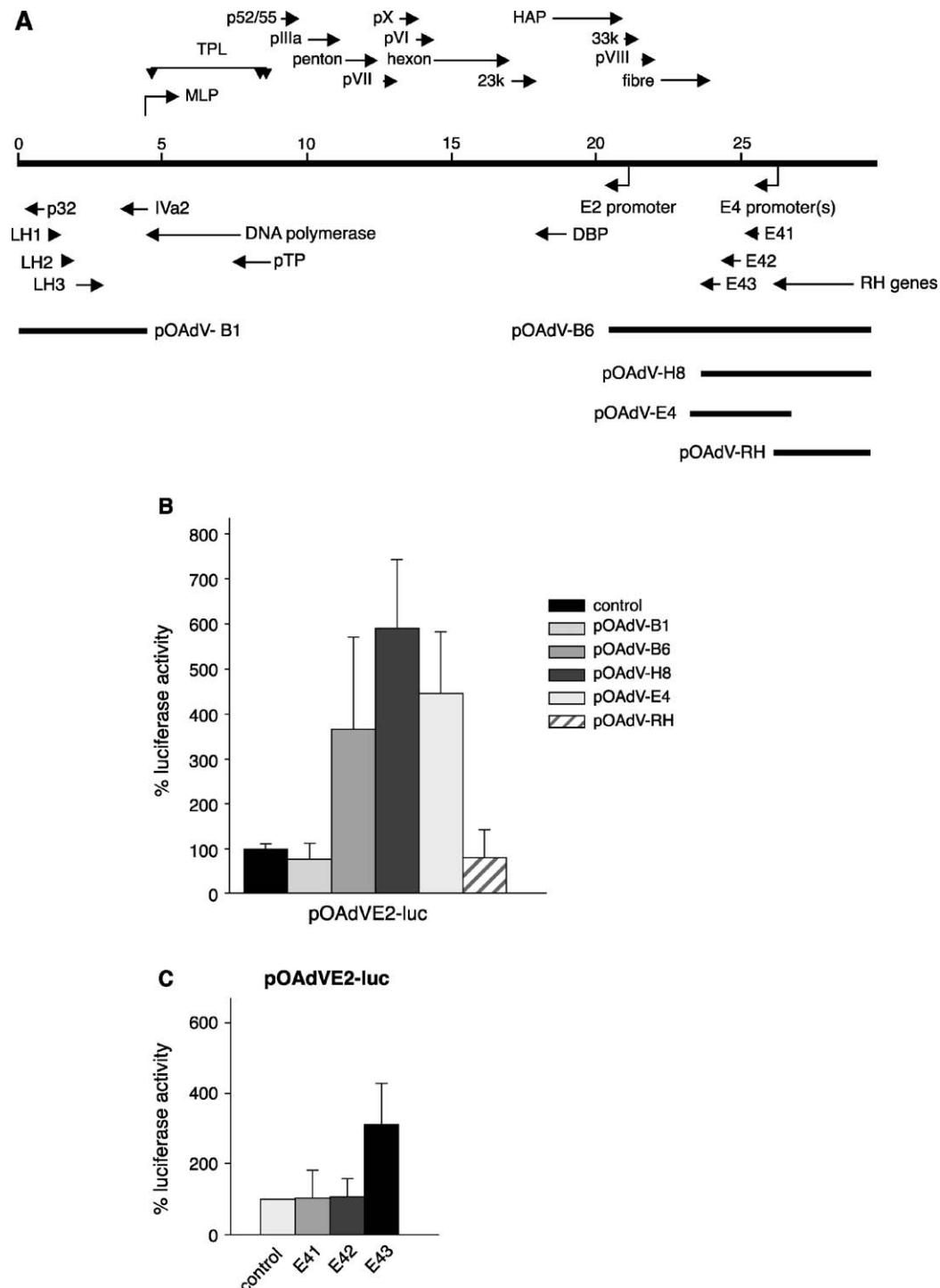


Fig. 2. Stimulation of the OAdV E2 promoter activity with different portions of the OAdV genome. (A) Schematic organization of the OAdV virus genome and regions of the OAdV genome subcloned as effector plasmids. (B) Reporter constructs (0.5 μ g) were co-transfected into CSL503 cells with transfection control plasmid pRSV-lacZ (0.1 μ g). For co-transfection assays, 0.4 μ g of the following plasmids was additionally transfected: pOAdV-B1 (left-hand end *Bam*HI fragment; nt 1–4253 of the OAdV genome), pOAdV-B6 (right-hand end *Bam*HI fragment; nt 20,862–29,574), pOAdV-H8 (right-hand end *Hpa*I fragment; nt 23,585–29,574), pOAdV-E4 (nt 23,327–26,985) and pOAdV-RH (nt 25,965–29,574). (C) Reporter constructs (0.5 μ g) were co-transfected with transfection control (0.1 μ g). For co-transfection assays, expression plasmids pCI-E41, pCI-E42, pCI-E43 (0.13 μ g) were additionally transfected. Reactions were supplemented with pCI to 1 μ g total DNA. For B and C, transfection samples were analyzed for reporter gene expression at 48 h as described in Fig. 1. Error bars indicate standard deviations from five independent experiments.

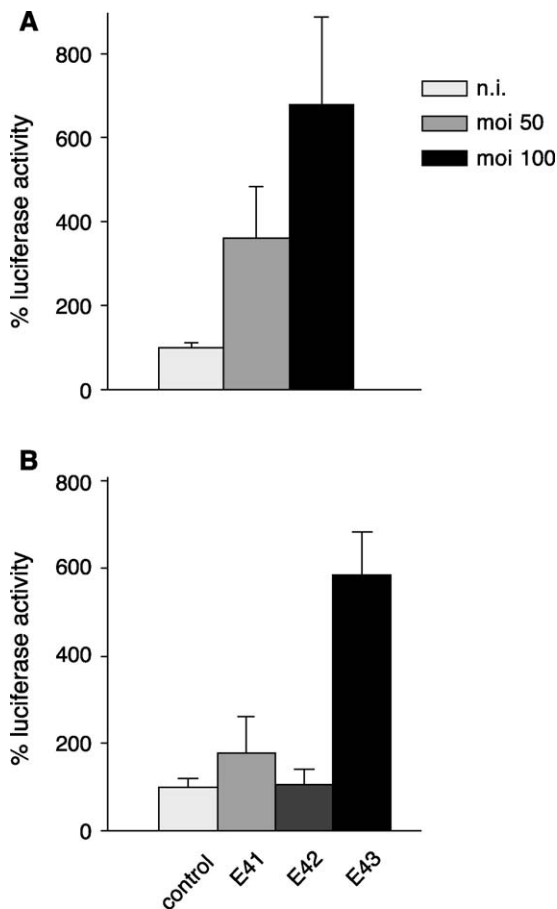


Fig. 3. Infection with OAdV or expression of E43 induces E2F-dependent gene expression. (A) Reporter plasmid pE2F-luc (0.8 μ g) and transfection control plasmid pRSVlacZ (0.2 μ g) were co-transfected into CSL503 cells. At 4 h post-transfection, cells were infected with OAdV at increasing multiplicity of infection (moi). At 30 h post-infection, cells were harvested and reporter gene activities were determined. Error bars indicate standard deviations from five independent experiments. n.i., non-infected. (B) pE2F-luc (0.5 μ g) was co-transfected with transfection control (0.1 μ g). For co-transfection assays, 0.13 μ g of the either pCI-E41, pCI-E42, pCI-E43 was additionally transfected. Reactions were supplemented with pCI to 1 μ g total DNA. At 48 h post-transfection, samples were analyzed for reporter gene expression as described in Fig. 1. Error bars indicate standard deviations from three independent experiments.

the highest amount of E2F plasmid (Fig. 4). To confirm that activation of the E2 promoter was dependent on the integrity of the E2F site within the OAdV E2 promoter, we mutated the binding site by site-directed mutagenesis (Fig. 5A) producing plasmid pOAdVmE2-luc. Co-transfection of this plasmid together with 100 ng of pCMV-E2F-1 diminished the E2F-mediated activation of reporter gene expression to 2.3-fold over control levels versus a 7.4-fold increase in the respective control experiment with the unmutated plasmid (Fig. 5B). The residual activation observed in the presence of E2F with the mutated promoter is probably caused by the complex nature of the viral E2 promoter which is not solely E2F dependent but is also stimulated by other factors which

might be activated by high E2F1 concentrations. In addition to the direct E2F1 effect, the binding site mutation also completely abolished the ability of E43 to stimulate E2 promoter activity when the E43 expression plasmid or pOAdV-B6 were used as effectors (Fig. 5C). This result indicates that E43 might perform its effect on the E2 promoter via E2F-1. To test this, we co-transfected the reporter plasmids pOAdVE2-luc or pE2F-luc and the effector plasmid pCMV-E2F-1 with or without the E43 expression plasmid into CSL503 cells. E2F activation of both the OAdV E2 promoter and the artificial E2F-dependent promoter was further increased by a factor of approximately 2.5 following co-transfection of pCMV-E2F1 and pCI-E43 (Fig. 6). This indicated that the E43 gene product might enhance the action of E2F-1 on both promoters.

E43 does not bind to pRb, but associates with E2F

We next wished to gain some insight into the nature of the possible interaction between the E43 gene product and E2F-1. In the case of HAdV5, the products of two genes are involved in induction of E2F activity: whereas the E1A gene products interact with the pRb protein, E4orf6/7 directly binds to E2F-1. We therefore attempted to detect a possible protein–protein interaction between E43 and pRb. Using extracts from 293 cells, which stably express the E1a gene, we successfully co-precipitated pRb with an antibody directed against HAdV5 E1A. A similar experiment was performed in which recombinant E43 protein purified from *Escherichia coli* as a Strep-tag II fusion protein was mixed with extracts from human 293 or ovine HVO156 cells, respectively, and precipitated with Strep-

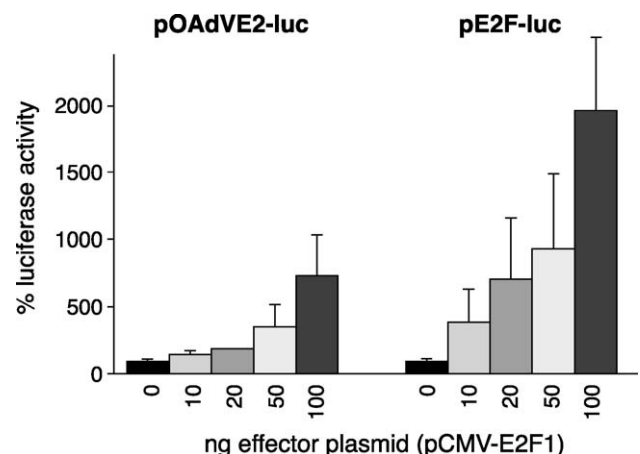


Fig. 4. The OAdV E2 promoter is activated by E2F. Reporter plasmids pOAdVE2-luc or pE2F-luc (0.7 μ g), respectively, were co-transfected into CSL503 cells with increasing amounts of effector plasmid pCMV-E2F1 and transfection control plasmid pRSVlacZ (0.1 μ g). DNA present in each reaction was equalized to 1 μ g with pBSKS⁺. At 48 h post-transfection, cells were harvested and reporter gene activities were measured as in Fig. 1.

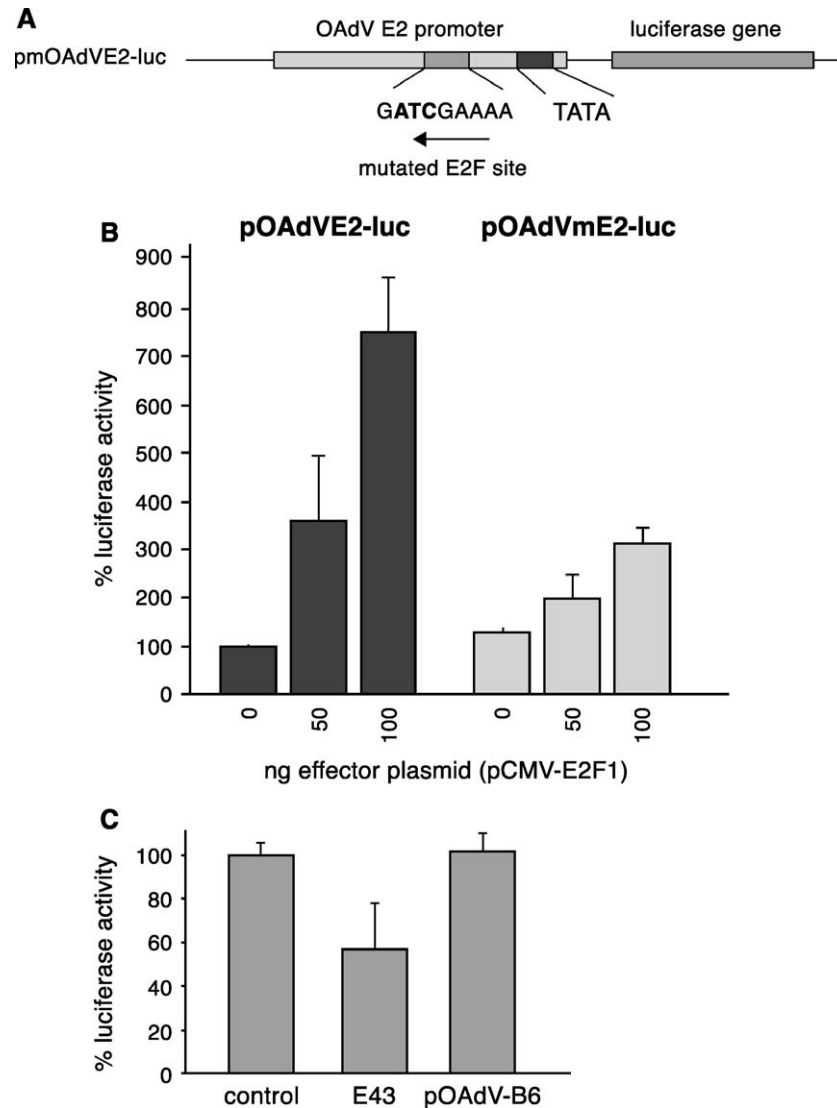


Fig. 5. Mutation of the E2F site reduces E2F dependence of the E2 promoter. (A) Scheme of reporter plasmid pOAdVmE2-luc containing the mutated E2F site. The altered nucleotides are given in bold. (B) CSL503 cells were transfected with pOAdVE2-luc or pOAdVmE2-luc (0.7 μ g), respectively, together with the transfection control pRSVlacZ (0.1 μ g) and pCMV-E2F1 (0.05 or 0.1 μ g). Reactions were supplemented to 1 μ g of total DNA with pBSSK⁺ where necessary. At 48 h post-infection, cells were analyzed for reporter gene expression as described in Fig. 1. Error bars indicate standard deviations from five independent experiments. (C) CSL503 cells were co-transfected with pOAdVmE2-luc (0.5 μ g), the transfection control pRSVlacZ (0.1 μ g) and either 0.4 μ g pCI-E43 or pOAdV-B6. At 48 h post-infection, cells were harvested and reporter gene activities were determined as described in Fig. 1. Error bars indicate standard deviations from four independent experiments.

Tactin sepharose. However, pRb could not be precipitated in association with E43 from either cell extract despite several attempts (data not shown), suggesting a lack of direct interaction between E43 and pRb. The idea that interaction with pRb is not a major mechanism of E43 action was also supported by preliminary findings that E43 could exert its effect on E2F-dependent expression in human cell lines that do not express a functional pRb protein (data not shown).

We next sought evidence for a direct interaction between the E43 and the E2F-1 proteins. Recombinant E43 fused to the *Strep*-tag II was mixed with cell extracts from either ovine HVO156 or human HeLa cells and

proteins were precipitated with *Strep*-Tactin sepharose. An equivalent fusion of the E41 protein was used as a control. In precipitates from extracts of both cell lines, we clearly detected E2F-1 after precipitation with the E43, but not with the E41 fusion protein (Fig. 7A), indicating binding between E43 and E2F-1. This interaction was specific as it depended on the presence of the E43 protein. Unfortunately, we could not precipitate E43 from infected cells due to a lack of a suitable combination of an E43-specific antibody and an antibody specific for the ovine E2F-1 gene product. However, addition of in vitro synthesized, ³⁵S-labeled E43 to lysates from either HVO156 or HeLa cells allowed precipitation of E43 with

a rabbit antibody directed against the human E2F-1 protein which cross-reacted with the ovine protein (Fig. 7B). Precipitation was not observed with a pre-immune serum or when using radiolabeled E41 protein instead of E43 (data not shown). Taken together, our data suggest that OAdV E43 probably does not bind to pRb but can bind directly to E2F-1.

Mutation of the OAdV E43 gene severely affects virus growth

If E43 interacts directly with E2F-1 and other family members, the gene may be important for modulating the cell cycle to establish optimal conditions for virus growth. To test this, we introduced an *Xba*I site at position 24,519 of the OAdV genome thereby introducing a stop codon early in the E43 open reading frame. The mutated OAdV genome was transfected onto the producer cell line HVO156 and recombinant viral clones were rescued between days 12 and 18 following transfection. The presence of the mutation was verified by PCR amplifying and sequencing the E4 region from three viral clones. However, on propagation, the mutated viruses showed severely delayed replication on both of the currently available producer cell lines (CSL503 or HVO156), despite several attempts. The best clone only grew to a titer of less than 10^8 viral particles (vp)/ml, whereas wild-type virus produced titers of about 10^{12} vp/ml. This finding demonstrates that E43 plays an important role in the viral life cycle and, although initially

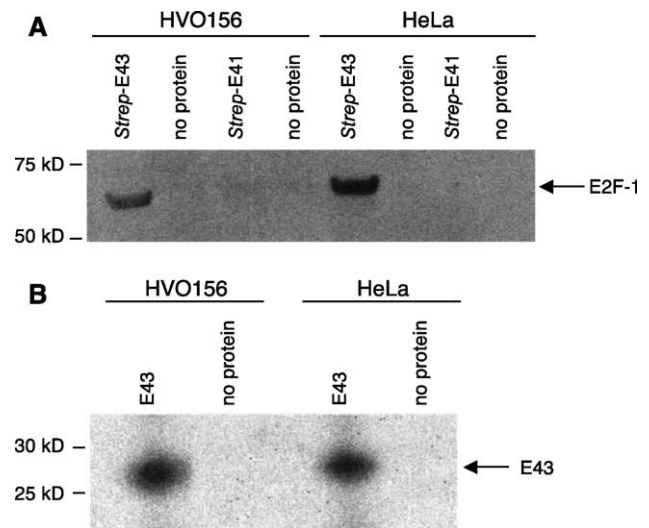


Fig. 7. Detection of binding between OAdV E43 and E2F. (A) HVO156 or HeLa cell lysates were mixed with *Strep*-E43 or *Strep*-E41 fusion proteins, respectively, and proteins were precipitated using *Strep*-Tactin sepharose. E2F-1 associated with the fusion proteins was detected by Western blotting using a polyclonal rabbit anti-E2F-1 antibody. (B) Lysates from HVO156 or HeLa cells were mixed with ³⁵S-labeled E43 and proteins were precipitated with a polyclonal rabbit anti-E2F-1 antibody. After SDS-PAGE co-precipitated, radioactively labeled proteins were detected by autoradiography. Molecular size markers are shown on the left while specific bands are indicated on the right.

dispensable for virus rescue, is necessary for efficient virus multiplication.

Discussion

Expression of the E2 region that encodes genes essential for viral DNA replication is an early step in the adenovirus life cycle and requires activation of the E2 promoter(s). In some mastadenoviruses, E2 promoter activation is dependent on E2F factors that are activated by products of the E1A and E4 regions, although not all E2F motifs are capable of binding E2F (Schaley et al., 2000). As OAdV shares a broad homology with other adenoviruses within the E2 region (Khatri and Both, 1998; Vrati et al., 1996a), it was expected that OAdV E2 gene expression might also be dependent on E2F activation. However, the OAdV E2 promoter region does not contain the inverted E2F binding sites that are present in the E2A promoters of mastadenoviruses such as HAdV2 and HAdV5. Instead, as identified here, it possesses a single copy of the consensus E2F binding sequence in close proximity to a TATA box. The atadenovirus genus is also notable for the apparent absence of gene homologues that could be involved in cell cycle deregulation during infection. Specifically, there is no E1A homologue and only minimal homology with one (orf6) of the seven E4 genes of HAdV2 and 5 (Täuber and Dobner, 2001). Similarly, there are no apparent equivalents of the GAM-1 or orf22 genes of the aviadenoviruses,

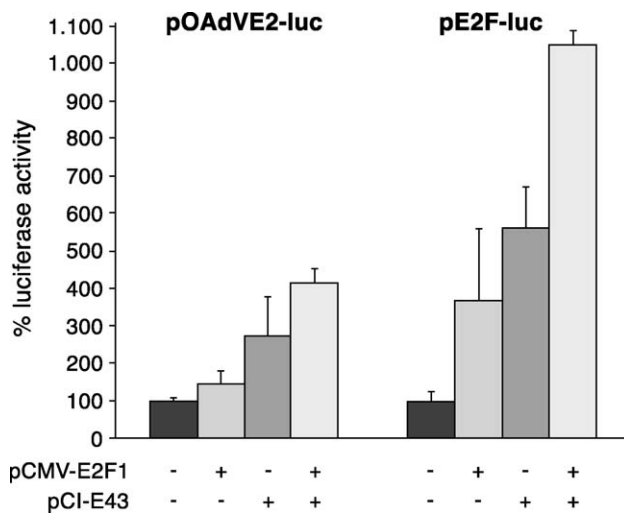


Fig. 6. Combined effect of E2F-1 and OAdV E43 on the E2F-dependent promoters. Reporter plasmids pOAdVE2-luc or pE2F-luc (0.5 μ g) were co-transfected with transfection control plasmid (0.1 μ g). pCMV-E2F1 (10 ng) and pCI-E43 (0.2 μ g) were additionally transfected either alone or in combination. Mixtures were supplemented with background plasmids pCMV, pCI and pGEM3 to 1 μ g total DNA where necessary. At 48 h post-transfection, samples were analyzed for reporter gene expression as described in Fig. 1. Error bars indicate standard deviations from three independent experiments.

although these, in turn, lack homology with E1A but are capable of binding the same pRb target (Lehrmann and Cotten, 1999).

A screen of OAdV for genes that were capable of activating the OAdV E2 promoter identified viral protein E43. The E43 gene product was found to induce expression from the OAdV E2 promoter as well as from an artificial E2F-dependent promoter. So far no evidence has been found that E43 interacts directly with pRb to release E2F as has been described for E1A proteins of mastadenoviruses (reviewed in Nevins, 1995). Therefore, E43 may be functionally distinct from these transforming proteins and others such as SV40 large T antigen or papillomavirus E7 that bind directly to pRb and related proteins. This concept is also supported by the observation that, in contrast to HAdV5, OAdV showed no transforming activity in a standard transformation assay involving rodent cells (Xu et al., 2000). Instead, we found that E43 binds directly to E2F-1, as has also been found for the HAd5 E4orf6/7 protein (Hardy and Shenk, 1989; Hardy et al., 1989; Huang and Hearing, 1989; Marton et al., 1990). It is also notable that E43 is so far the only atadenovirus gene product that has been shown to interact with a host cell protein.

The underlying mechanism of E2F-dependent promoter activation by E43 is not clear. E4orf6/7 has been shown to direct E2F dimerization that is critical for activation of cellular E2F-dependent promoters (Obert et al., 1994). Whether E43 acts in this way is presently unknown. The sequences involved in E4orf6/7 interaction with E2F-1 and the binding site on E2F-1 have been identified (reviewed in Täuber and Dobner, 2001). However, there is no detectable homology between E4orf6/7 and E43. The only motif within the E43 protein with homology to a known protein is a cysteine-rich motif (CCR) that is also present in E42 and all mastadenoviral E4orf6 proteins (Nevels et al., 2000; Vrti et al., 1996b). Cellular proteins that also contain this motif are known to interact with E2 ligases of the ubiquitin-dependent proteolytic system (Lorick et al., 1999). However, at this stage, it is not clear if this motif is of any importance to the function of E43 in stimulating E2F-dependent gene expression and in the potential functional homologue, E4orf6/7, this motif is removed by splicing.

It is important to note that our approach did not identify an E2 promoter-activating gene nor any E2F inducing activity within the left-hand region of the OAdV genome where initially, a functional homologue of E1A was expected to reside. This again emphasizes the fundamental differences between mast- and atadenoviruses. Similarly, a screen of the CELO genome, an aviadenovirus, identified Orf 22 and GAM-1 as two genes within the right-hand quarter of the genome whose products were capable of pRb binding and thus, E2F activation (Lehrmann and Cotten, 1999). It is therefore apparent that adenovirus genera have evolved multiple strategies to activate E2F transcription factors to initiate viral gene expression and replication. It

remains to be determined whether other atadenovirus genes are also involved.

It has been shown by phylogenetic analysis of DNA polymerase genes that adenoviruses, as typified by OAdV, are older than viruses in other adenovirus genera and closely related to reptilian adenoviruses (Benkö et al., 2002). Therefore, E43 may represent an early version of viral proteins with capacity to interfere with the E2F pathway. Other adenoviruses might have acquired different, additional and/or partially redundant functions for E2F deregulation over time. Although they lack obvious homologues, it will be interesting to determine whether other phylogenetically older adenoviruses such as members of the siadenovirus and the proposed ichtadenovirus genera also contain gene products capable of E2F interactions.

Materials and methods

Cells and viruses

CSL503 (fetal ovine lung) (Pye, 1989) and HVO156 (fetal ovine skin) (Löser et al., 2002) cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Karlsruhe, Germany) plus 15% fetal calf serum (FCS, BioWest, Nuaille, France). Both cell lines are permissive for OAdV replication. HeLa cells (ATCC CCL-2) were grown in DMEM and 10% FCS. Media were generally supplemented with 2 mM glutamine, 100 µg/ml streptomycin and 100 units/ml penicillin.

Wild-type OAdV was propagated as described (Löser et al., 2001). The number of viral particles (vp/ml) was determined according to the method of Maizel et al. (1968) by measuring the absorption at 260 nm. The number of infectious particles (ip/ml) was assayed by limiting dilution on CSL503 cells as described elsewhere (Cichon et al., 1999). The vp/ip ratios were generally <20.

Infection of cells with OAdV was performed in six-well plates at the multiplicity indicated in the Results section and following the method described elsewhere (Kümin et al., 2002).

Transfections and luciferase assays

Transfections of CSL503 cells were carried out using the Lipofectamine Plus™ kit (Invitrogen) according to the manufacturer's instructions. Cells were seeded 1 day before transfection in six-well plates at a density of 3×10^5 per well. Total plasmid DNA (1 µg) in serum-free medium was used. To normalize transfection data, pRSVlacZ (0.1 µg) was used for co-transfection in each experiment, and luciferase and lacZ gene activities were determined in the same cell lysate.

Luciferase assays were performed following the method described (Löser et al., 1996). Briefly, 24–48 h post-transfection/infection, cells were washed once in PBS and

lysed in 300 µl lysis buffer [100 mM potassium phosphate (pH 7.8), 1 mM DTT, 1% Triton X-100] for 5 min. The lysate was centrifuged to remove cell debris and a portion of the supernatant (50 µl) was added to reaction buffer (180 µl) [25 mM K₂PO₄ (pH 7.8), 4 mM EGTA, 15 mM MgSO₄, 1 mM ATP, 1 mM DTT]. Luciferase activity was measured in a LB9507 luminometer (Berthold, Bad Wildbad, Germany) in the presence of 18 µM luciferin (Sigma, Taufkirchen, Germany) for 10 s. Results were normalized to the lacZ gene activity in the same cell lysate.

The quantification of the lacZ gene activity was also performed with a luminometer using the Galakto-Light™ kit (Tropix, Bedford, USA) according to the manufacturer's instructions.

Plasmids

Plasmid pE2F-luc which is based on pGL2-Basic (Promega, Mannheim, Germany) and carries three E2F consensus binding sites (CGC CGC) was kindly provided by Christian Hagemeyer (Berlin, Germany). pCMV-E2F1 was kindly provided by Kristian Helin (Helin et al., 1993). The OAdV E2 promoter was amplified by PCR using primers 5'GCAACTGCAGAAGAACTGTTCCAGTATC 3' and 5'GCGGGATCCGCCAGTTGTTGATGTTAACC 3' and cloned upstream of a luciferase reporter gene in pGL2-Basic, giving rise to pOAdVE2-luc. Plasmid pRSV-lacZ contains the *E. coli* lacZ gene flanked by the RSV LTR and the bovine growth hormone polyA-signal. The left-hand *Bam*HI fragment (nucleotides 1–4253) of OAdV was cloned as an *Asp*718/*Bam*HI fragment from pOAV3-cos (Löser et al., in press) into pBSKS[−] (Stratagene, Heidelberg, Germany) giving rise to pOAdV-B1. pOAdV-B6 contains the right-hand end *Bam*HI fragment (nucleotides 20,862–29,574 of the OAdV genome) and was cloned as a *Bam*HI/*Asp*718 fragment into pBSKS[−], while pOAdV-H8 contains the right-hand end *Hpa*I fragment (nucleotides 23,585–29,574 of the OAdV genome) cloned as *Hpa*I/*Asp*718 into the same vector. pOAdV-E4 (nucleotides 23,327–26,985) was obtained by *Pst*I/*Hind*III digest of pOAdV-B6 and subsequent insertion into pBSKS[−] (Stratagene), while pOAdV-RH (nucleotides 25,965–29,574) was generated by cutting pOAdV-H8 with *Spe*I and ligation of the cleavage product into pBSKS[−]. E41, E42 and E43 from OAdV were PCR-cloned without introns into pCI (Promega) using primers 5'CGGGGTACCATGAATCAACATGGGAGAAT 3' and 5'CGGGGTACCTCACTGCCAATCAGGCTT 3', 5'CGGGGTACCATGGATAAGCAAATCAAAGTT 3' and 5'CGGGGTACCGAGTTCATGCTACATCCAA 3', or 5'CGGGGTACCATGAACTCCATTATGTGTTG 3' and 5'CGGGGTACCTTAGCTGAAAATGTACCAGT 3', respectively.

For expression of E41 and E43 in *E. coli*, both genes were PCR amplified using primers 5'GGAATTC

CCAATCAACATGGGAGAATGCT 3' and 5'CAAC TGCAGTCACTGCCAATCAGGCTTAA 3', or 5'GGAATTCCAACTCCATTATGTGTTGCGA 3' and 5'CAACTGCAGTTAGCTGAAAATGTACCAGT 3', respectively, and cloned into pASK-IBA5 (IBA GmbH, Göttingen, Germany) to give rise to pIBA5-E41 and pIBA5-E43. pGEM3-E43 and pGEM3-E41 for *in vitro* transcription/translation were cloned by excising the gene from pCI-E43 with *Eco*RI and *Bam*HI and inserting it into pGEM3 (Promega).

Mutation of E2F binding site in the OAdV E2 promoter reporter gene construct

Using primers 5'CTGGTTGAGCCATGGATCGAAA ATTTAAACTATAAAGCCTCAGC 3' and 5'GC TGAGGCTTTATAGTTTTTAAATTTTCGATCCATG GCTCAACCAG 3' (mutated nucleotides are shown in bold), the putative E2F binding site in the E2 promoter was mutated in pOAdVE2-luc using the QuickChange™ site-directed mutagenesis kit (Stratagene).

Mutation of the E43 ORF of OAdV and rescue of mutated virus

A stop codon was introduced immediately downstream of the start codon of E43 by prior sub-cloning of the E4 region as an *A*fIII fragment. Following this, an *Acc*I/*Hpa*I segment within the OAdV *A*fIII fragment was further sub-cloned into pBSSK[−]. Using primer pair 5'GATTCATCATCTTTTTTATTCTAGAGTGATATACC GTTTGTTAG 3' and 5'CTAACAAACGGTATATCAC TCTAGAAATAAAAAAGATGATGAATC 3' (mutated nucleotides shown in bold) and the QuickChange™ site-directed mutagenesis kit (Stratagene), a *Xba*I site was introduced at position 24,519 of the OAdV genome to produce a stop codon after amino acid 15 of the E43 open reading frame. The mutated fragment was then re-introduced into the original plasmid and virus was rescued as described (Löser et al., 2001).

Production of recombinant proteins in *E. coli*

E41 and E43 were expressed in *E. coli* BL-21 (Stratagene) from pIBA5-E41 and pIBA5-E43, respectively, using the *Strep*-tag kit (IBA) according to the manufacturer's instructions.

Immunoprecipitations of proteins

Cells were seeded in 6-cm dishes and harvested when approximately 90% confluent utilizing solutions provided in the Protein A immunoprecipitation kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Proteins were recovered with either 1 µg specific antibody and protein A agarose or in association with 20 µg

Strep-tagged protein purified from *E. coli* and *Strep*-Tactin sepharose (IBA) and analyzed by Western blotting. E1A and polyclonal rabbit anti-E2F-1 antibodies were obtained from Santa Cruz (Heidelberg, Germany). A mouse monoclonal antibody directed against human pRb was obtained from Pharmingen (Heidelberg, Germany).

E43 was synthesized from the pGEM3 plasmids and labeled with ^{35}S -methionine (Amersham, Freiburg, Germany) using the TnT® Coupled Wheat Germ Extract System (Promega) according to the manufacturer's instructions. Five microliter-samples were separated on 4–12% Bis–Tris gels (Invitrogen) to confirm correct synthesis of the proteins. Gels were then fixed in 50% (v/v) methanol, 10% (v/v) glacial acetic acid for 30 min and soaked in Amplify (Amersham) for 15 min, dried and exposed on Kodak BioMax MR films overnight. Radiolabeled E43 was mixed with cell lysates and precipitated with E2F utilizing the Protein A immunoprecipitation kit. Proteins were released by boiling the beads in 2× Laemmli sample buffer for 5 min, separated by SDS-PAGE and visualized by autoradiography.

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